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Palmitoylation of a pulmonary surfactant protein C analogue affects the surface associated lipid reservoir and film stability

M. Gustafsson [a], M. Palmblad [b], T. Curstedt [b], J. Johansson [a,*], S. Schürch [c]

[a] *Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-171 77 Stockholm, Sweden*
[b] *Department of Clinical Chemistry, Karolinska Hospital, Stockholm, Sweden*
[c] *Department of Physiology and Biophysics, University of Calgary, Calgary, Alta., Canada*

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Abstract

Surfactant protein C (SP-C) is a lipopeptide that contains two thioester-linked palmitoyl groups and is considered to be important for formation of the alveolar surface active lipid film. Here, a non- or dipalmitoylated SP-C analogue (SP-C(Leu)), in which all helical Val residues were replaced with Leu and Cys-5 and Cys-6 were replaced with Ser, was tested for surface activity in a captive bubble system (CBS). SP-C(Leu), either palmitoylated at Ser-5 and Ser-6 or non-palmitoylated, was added to mixtures of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)/phosphatidyl glycerol (PG)/palmitic acid (PA), 68:22:9, (by mass) at a concentration of 2 and 5%. With 2% peptide, surface film formation was rapid, reaching a surface tension below 25 mN/m within 5 s, but the samples with 5% SP-C(Leu) required more than 20 s to reach values below 25 mN/m. Minimum surface tension for the samples with dipalmitoylated SP-C(Leu) was below 1.5 mN/m and very stable, as the surface tension increased by less than 0.5 mN/m within 10 min at constant bubble volume. Minimum surface tension for the non-palmitoylated SP-C(Leu) was approximately 2 and 5 mN/m for 2 and 5% peptide, respectively, but the films were less stable as seen by frequent bubble clicking at low surface tensions. Films with dipalmitoylated SP-C(Leu) that were dynamically cycled at 20–30 cycles/min were substantially less compressible at a surface tension of 20 mN/m (0.007 m/mN) than those that contained the non-palmitoylated peptide (0.02 m/mN). After subphase depletion, the incorporation of lipids into the surface active film during initial bubble expansion occurred at a relatively low surface tension (about 35 mN/m) for the samples with dipalmitoylated SP-C(Leu) compared to approximately 45 mN/m for those containing the non-palmitoylated peptide. Furthermore, for samples that contained non-palmitoylated SP-C(Leu), the ability to reach near zero stable surface tension was lost after a few adsorption steps, whereas with the dipalmitoylated peptide the film quality did not deteriorate even after more than 10 expansion steps and the incorporation of reservoir material equivalent to more than two monolayers. It appears that the covalently linked palmitoyl groups of the SP-C analogue studied are important for the mechanical stability of the lipid film, for the capacity to incorporate material from the reservoir into the surface active film upon area expansion, and for the low film compressibility of dynamically cycled films.

Keywords: Protein acylation; Captive bubble surfactometer; Synthetic peptide

Abbreviations: BLES, bovine lipid extract surfactant; CBS, captive bubble surfactometer; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; TFA, trifluoroacetic acid; SP, surfactant protein; PA, palmitic acid; PG, phosphatidyl glycerol

* Corresponding author. Fax: +46-8-337462; E-mail: jan.johansson@mbb.ki.se

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1. Introduction

The structural characterization of the surfactant associated protein C (SP-C) has been advanced mainly by the fact that SP-C is considered an important component of surfactant preparations used for replacement therapy for the respiratory distress syndrome (RDS) in premature infants. Most of the commercially available surfactants contain the hydrophobic proteins SP-B and SP-C, and are obtained by extraction from animal lungs. Such procedures are relatively demanding, and propagation of infectious material cannot be excluded. Since the structure of SP-C, a 35-residue lipopeptide with two palmitoyl chains is now known to a significant extent [1], SP-C analogues can be synthesized with the aim to replace the present generation of animal-derived surfactant preparations.

The primary structure of SP-C is evolutionarily conserved and it appears to be present only in pulmonary surfactant, suggesting that it might have important and specific functions. The structure of SP-C and its structure–activity relationship in a phospholipid environment has been studied. The three-dimensional structure in an aqueous mixed organic solvent revealed one continuous 37-Å-long α-helix encompassing residues 9–34 as the only regular structural element. The central 23 Å of the helix contains exclusively aliphatic residues with branched side-chains, mainly valines, and exposes an all-hydrophobic regular surface. The size of the entire helix perfectly matches the thickness of a fluid DPPC membrane, and the all-hydrophobic part of the helix matches the acyl-chain part of such a bilayer [2]. This supports a transmembrane orientation of SP-C in pulmonary surfactant bilayers [3,4]. In a phospholipid monolayer, the SP-C helix is tilted, thereby maximizing the interactions with the lipid acyl-chains also in this environment [5]. SP-C contains palmitoylcysteines at positions 5 and 6 that appear to be important both for the integrity of the α-helical structure and for functional properties [1]. Since the conformation of the N-terminal part in a phospholipid environment is not known, the mechanisms whereby the SP-C thioester-linked palmitoyl chains affect structure and function remain to be determined.

Impaired surface activity, including reduced adsorption and reformation of the surface film at the air–water interface, reduced mechanical stability and increased film compressibility, was observed for phospholipid combinations with chemically depalmitoylated SP-C [6,7]. These studies indicated that palmitoylation might be important for optimal surface activity of SP-C. One problem associated with studies on chemically depalmitoylated SP-C molecules is that they are less helical than the native lipopeptide, indicating structural rearrangement [1]. Furthermore, synthetic peptides with the amino acid sequence of SP-C are inefficient in folding into a helical conformation [8] and the yields of dipalmitoylated SP-C expressed in baculovirus are at present not sufficient to allow extensive studies [9]. Recently an SP-C analogue, SP-C(Leu) with all helical valine residues in native SP-C replaced with leucine and the palmitoylcysteines at positions 5 and 6 replaced with serine was synthesized [8]. The serines were introduced in order to prevent disulfide-dependent oligomerization. This SP-C analogue had a similar α-helical content and transmembrane orientation as native SP-C, but in contrast to poly-valyl-containing synthetic peptides, non-palmitoylated SP-C(Leu) folds into a helical conformation after acid-induced denaturation. The latter correlates with the helix versus sheet propensities of Val and Leu; Val is overrepresented in β-sheet structures while Leu favors helix formation. The ability of SP-C(Leu) to refold after acidic treatment allows chemical palmitoylation of the peptide after synthesis [10]. Non-palmitoylated SP-C(Leu) added to DPPC/PG/PA, (68:22:9, by mass), increased the surface activity of that mixture as tested by film formation upon spreading using a Wilhelmy balance. In addition, it has the ability to lower the surface tension to near zero values in a pulsating bubble surfactometer [8].

For the present study, dipalmitoylated SP-C(Leu) was synthesized by linking palmitoyl chains to Ser-5 and Ser-6 [10]. The objective has been to investigate the influence of palmitoylation of SP-C(Leu) by studying the surface activity of peptide/lipid mixtures in a captive bubble surfactometer (CBS) [11,12]. The tests in the captive bubble also included the study of the surface associated surfactant reservoir [13]. We found that palmitoylation is important for the incorporation of surfactant material upon film expansion and for the mechanical stability of the highly com-

pressed film at near zero minimum surface tension. In addition, palmitoylation of SP-C(Leu) appears to be important for the periodic reformation of the surface film of dynamically cycled films.

2. Materials and methods

2.1. Materials

DPPC, egg PG, PA were purchased from Sigma and were used without further purification. *tert*-butyloxycarbonyl (*t*-BOC) amino acids and reagents for peptide synthesis were from Perkin-Elmer.

2.2. Synthesis of SP-C(Leu) and dipalmitoylated SP-C(Leu)

SP-C(Leu), with the amino acid sequence FGIPSSPVLKRLLILLLLLLLILLLILGALLMGL, was synthesized by solid-phase techniques and *t*-BOC chemistry using an ABI 430A instrument as described [8]. After deprotection and extraction of scavengers and protecting groups, the peptide was purified by reversed-phase HPLC (Vydak C18, 22×250 mm) and a linear gradient of 2-propanol in 75% ethanol over 40 min with a flow rate of 7 ml/min (both solutions containing 0.1% TFA) [14]. For synthesis of *O,O*-dipalmitoylated SP-C(Leu) [10], SP-C(Leu) was dissolved in distilled TFA and a 20-fold molar excess of distilled palmitoyl chloride was added. After 10 min, the reaction was quenched with 80% ethanol. Purification was performed by chromatography over Lipidex-5000 in ethylene chloride/methanol 1:4 (v/v) to remove excess of palmitic acid, followed by reversed-phase HPLC as for the non-palmitoylated peptide. The correct molecular mass of the product was verified by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry.

2.3. Reconstitution of peptides and lipids

Aliquots of the lipids were dissolved in chloroform/methanol, 98:2 (v/v), and non-palmitoylated SP-C(Leu) or dipalmitoylated SP-C(Leu) in chloroform/methanol, 1:1 (v/v), were added from stock solutions in which peptide concentrations had been determined by amino acid analysis. The solutions were then aliquoted into samples containing 1.2 mg of DPPC/PG/PA, (68:22:9 by mass) and either 2 or 5% of peptide (by mass of the lipids). The samples were then dried under nitrogen and stored at −20°C until used. Before use, samples were hydrated at a final lipid concentration of 1 mg/ml with a buffered salt solution of 0.9% NaCl, 2.0 mM $CaCl_2$, 10 mM HEPES, pH 6.9, and incubated at 37°C for 10 min.

2.4. Surface activity measurements

2.4.1. Apparatus

A captive bubble surfactometer [11,12,15] was used to measure lipid adsorption, the surface tensions and the corresponding film areas of bubbles compressed and expanded under quasi-static or dynamic conditions, and the film compressibility at the surface tension of 20 mN/m for dynamically cycled films.

2.4.2. Adsorption studies

While stirring, an atmospheric bubble with a diameter of 6–7 mm was introduced into the sample chamber. The bubble took its resting position shape within the time corresponding to six video frames or 0.2 s. This was considered *time zero* for adsorption, after which, the bubble was left undisturbed for 5 min. Changes in surface tension due to surfactant adsorption resulted in changes in bubble shape. With the latter closely monitored using a video system, bubble diameters were measured from digitized images and surface tension, area, and volume of the captive air bubbles were calculated [16]. Three independent measurements of adsorption followed by three series of four quasi-static cycles (see below) were performed using three chamber fillings of each lipid–peptide combination.

2.4.3. Quasi-static surface tension measurements

After an initial adsorption period of 5 min, the bubble was compressed stepwise and left after each step until bubble shape remained constant for a period of 20 s. When further compression does not result in further reduction in surface tension, as seen by no further reduction in the bubble's height, the bubble has reached its minimal surface tension.

Once a minimal surface tension was reached, the bubble was expanded stepwise to the original volume. In each series of quasi-static cycles, the process was repeated four times with the same bubble. Average surface tension values of the first and fourth cycles were plotted against relative bubble area, taken as 1.0 at the surface tension of 23.0 mN/m.

2.4.4. Dynamic surface tension–area cycling

After the initial 5-min adsorption period, the bubble was continuously compressed and expanded at a rate of 20–30 cycles/min for 20 cycles. Once a minimum surface tension is reached, further compression of a bubble results in the formation of a collapse phase, which might remain associated with the interface or be lost to the surrounding subphase [15]. This process is known as overcompression. In dynamic cycling experiments, bubbles were compressed to produce 5–10% collapse area in relation to the total film area compression. Minimum and maximum surface tensions were determined from 10 successive cycles centered on number 10 of the 20 dynamic cycles.

2.4.5. Subphase depletion

A bubble was formed and left to adsorb to the equilibrium surface tension of ~25 mN/m. The surfactant suspension in the sample chamber was then removed and substituted with 10 mM HEPES, 0.9% NaCl, 1.5 mM $CaCl_2$, pH 6.9, without disturbing the bubble. At least six volumes of fresh salt solution were introduced to ensure the removal of surfactant from the subphase. During this process, the surface tension at the bubble surface remained below 25 mN/m. The bubble was then compressed stepwise in quasi-static fashion until minimum surface tension was obtained [13]. Minimum surface tension is achieved when the bubble ceases to flatten and starts to shrink in its width upon volume compression in small steps. After reaching near zero minimum surface tension (about 1 mN/m for lipid extract surfactant) the bubble was expanded in quasi-static fashion to its original size, and then left for 2 min. The bubble was then further expanded stepwise to increase the diameter at each step by approximately 0.5 mm. After each step, a waiting period of 2 min was observed during which the bubble height decreases, indicating decreasing surface tension by adsorption from a 'surface associated surfactant reservoir' since adsorption cannot take place from the surfactant-depleted bulk phase [13]. After 3–4 adsorption steps, the bubble was compressed until minimum surface tension was obtained again. Bubble expansion in adsorption steps with waiting periods of 2 min were then continued as were compressions to minimum surface tension until stepwise bubble expansions was no longer accompanied by adsorption, and the surface tension began to rise rapidly to values substantially above the equilibrium surface tension. At this point, there is no further adsorption from the surface reservoir and the film expansion characteristics are consistent with that of a monolayer [13]. The bubble area at minimum surface tension measured on the first compression is then compared with the bubble area at minimum surface tension obtained after compression at maximum size of the bubble. With this approach, the material in excess of one monolayer, associated with the bubble surface, can be estimated [13].

2.4.6. Film compressibility calculations

Film compressibility at a particular surface tension, $C\gamma$, was calculated according to

$$C\gamma = (1/A)(\mathrm{d}A/\mathrm{d}\gamma) \quad (1)$$

where γ is a particular surface tension chosen, A is the area at that surface tension, $\mathrm{d}A/\mathrm{d}\gamma$ is the reciprocal of the slope of the curve at the particular surface tension. The film compressibility C_{20} at γ= 20 mN/m was calculated from four successive dynamic cycles centered on number 10 from a series of 20 cycles. A negative slope in the expansion part of a particular curve at low surface tensions indicates bubble clicking characteristic for film instability (e.g. Fig. 2B).

2.5. Statistical analysis

Where applicable, data points in the graphs represent means ± S.E.M. Multiple mean comparisons were done using a one-way analysis of variance in conjunction with the Newman–Keuls method.

3. Results

3.1. Adsorption

At the end of bubble formation (<0.2 s) the surface tensions reached by non- and di-palmitoylated preparations of SP-C(Leu) were between 31 and 34 mN/m, with no discernible differences ($P>0.1$). There was no difference in the adsorption rate and the surface tension after 5 min between the samples with dipalmitoylated and non-palmitoylated SP-C(Leu), both with 2 and 5% peptide. However, for the dipalmitoylated peptide, the surface tension reached on adsorption at and above 2 s was significantly lower for 2% SP-C (Leu) vs. 5% SP-C (Leu) (Fig. 1, Table 1) and at and above 5 s, the surface tension was also lower for 2 vs. 5% of the non-palmitoylated peptide (Table 1).

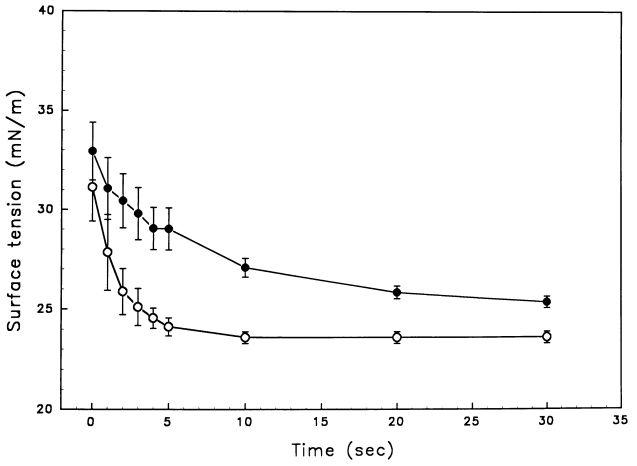

Fig. 1. Time course for adsorption of dipalmitoylated SP-C(Leu) in DPPC/PG/PA. Surface tension is plotted vs. adsorption time. Open circles, 2% peptide, filled circles, 5% peptide.

3.2. Quasi-static film compression and expansion cycles

The difference between the samples containing dipalmitoylated and non-palmitoylated SP-C(Leu) became more apparent when the film was compressed and expanded stepwise. Film compressibility determined at the surface tensions of 20 mN/m decreased progressively with quasi-static cycling and so did the surface tension vs. area hysteresis (Fig. 2). The minimum surface tension achieved was approximately 1 mN/m for the samples with 2% dipalmitoylated SP-C(Leu) (Fig. 2A, Table 1). In contrast, with 2% non-palmitoylated SP-C(Leu) the minimum surface tension obtained on the first quasi-static compression was $\sim$4 mN/m (Fig. 2B) and for all of the four quasi-static compressions of the films with 5% non-

Table 1
Statistical analysis of differences between surface properties of DPPC/PG/PA 68:22:9 (w/w/w), mixed with dipalmitoylated or non-palmitoylated SP-C (Leu)

	Dipalmitoylated		Non-palmitoylated		*P* values			
	(A) 2%	(B) 5%	(C) 2%	(D) 5%	A vs. B	A vs. C	B vs. D	C vs. D
Surface tension (mN/m)								
Adsorption								
at <0.2 s	31.1 ± 1.71	32.9 ± 1.45	33.3 ± 0.64	33.9 ± 1.73	NS	NS	NS	NS
at 1.0 s	27.8 ± 1.90	31.1 ± 1.56	29.7 ± 1.10	31.0 ± 2.29	NS	NS	NS	NS
at 2.0 s	25.9 ± 1.14	30.4 ± 1.36	28.2 ± 1.06	29.7 ± 1.79	<0.01	NS	NS	NS
at $\geq$5.0 s	24.1 ± 0.44	29.0 ± 1.06	24.3 ± 0.19	26.9 ± 0.05	<0.01	NS	NS	<0.01
Quasi-static cycles								
Minimum surface tension	1.1 ± 0.06	2.0 ± 0.29	2.0 ± 0.15	5.2 ± 0.15	<0.05	<0.01	<0.01	<0.05
Dynamic cycles								
Minimum surface tension	0.8 ± 0.23	1.0 ± 0.10	1.2 ± 0.08	3.1 ± 0.07	NS	NS	<0.01	<0.01
Maximum surface tension	40.9 ± 0.74	42.8 ± 0.31	44.4 ± 1.50	46.3 ± 0.18	NS	<0.01	<0.01	NS
Compressibility $\times 10^3$ (m/mN)								
Film compressibility								
On Compression at 20 mN/m	6.8 ± 1.23	12.0 ± 1.23	15.0 ± 1.20	14.0 ± 1.20	<0.01	<0.01	<0.05	NS

Values are means ± S.E.M. NS, not significant ($P>0.05$).

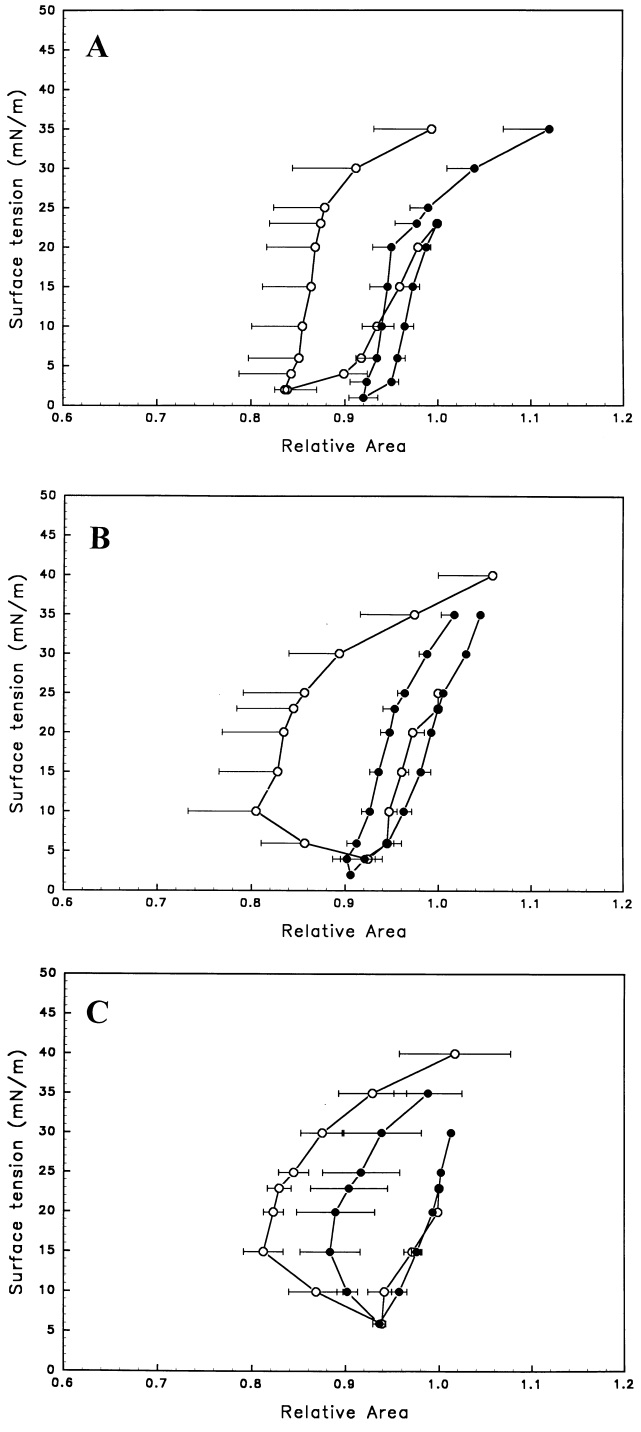

Fig. 2. Quasi-static isotherms of non-palmitoylated and dipalmitoylated SP-C(Leu). Surface tension is plotted vs. relative area for the first (open circles) and fourth (filled circles) quasi-static cycle. (A) 2% dipalmitoylated SP-C(Leu), (B) 2% and (C) 5% non-palmitoylated SP-C(Leu) in DPPC/PG/PA (68:22:9 by mass).

palmitoylated SP-C(Leu) it did not decrease below 5 mN/m (Fig. 2C). In addition, the films with the non-palmitoylated peptide were less stable, as seen by the negative slope for film expansion from the minimum surface tension (Fig. 2B,C). A negative slope of the surface tension–area relation is consistent with the concept of bubble clicking at near zero surface tension, which indicates film instability.

3.3. Dynamic film compression and expansion cycles

With 2% peptide the minimum surface tensions obtained during continuous cycling at 20–30 cpm were approximately 1 mN/m for the non-palmitoy-

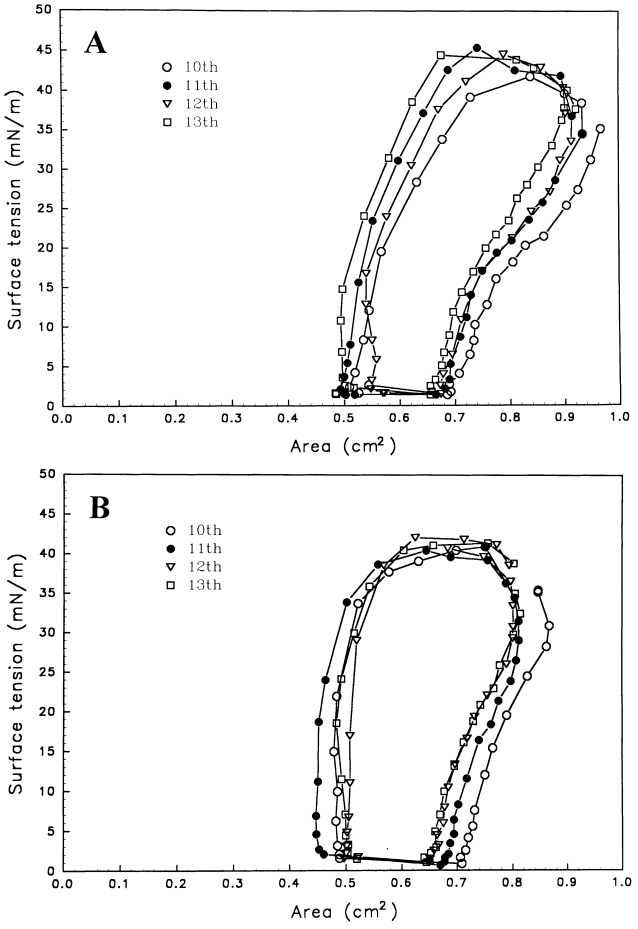

Fig. 3. Dynamic (20–30 cycles/min) surface tension–area relationships of non-palmitoylated and dipalmitoylated SP-C(Leu). Plots show four consecutive dynamic cycles; cycles 10 (open circles), 11 (filled circles), 12 (triangles), and 13 (rectangles). (A) 2% non-palmitoylated SP-C(Leu), (B) 2% dipalmitoylated SP-C(Leu) in DPPC/PG/PA (68:22:9 by mass).

lated and dipalmitoylated SP-C(Leu) (Fig. 3), but for the samples with 5% peptide, the non-palmitoylated SP-C(Leu) gave a minimum surface tension above 3 mN/m (Table 1). With 2% peptide, the film compressibility at $\gamma = 20$ mN/m was approximately 0.02 m/mN for the non-palmitoylated peptide compared to approximately 0.007 m/mN for the dipalmitoylated peptide (Table 1). This is consistent with the existence of a more distinct plateau at about 20 mN/m, as seen by the change in curvature, for the non-palmitoylated peptide compared to the very slight change in curvature for the dipalmitoylated peptide (Fig. 3A,B). The maximum surface tensions (expressed in mean ± S.D., $n = 4$) during dynamic cycling were lowest for the samples with 2% (40.9 ± 0.74 mN/m) and 5% dipalmitoylated SP-C(Leu) (42.8 ± 0.31) mN/m, but no difference was discernible at the 5% level of significance. The maximum surface tension was significantly lower for the dipalmitoylated peptide than for the non-palmitoylated one at both 2 and 5% (Table 1).

3.4. The surface associated surfactant reservoir

After subphase depletion, the incorporation of surfactant material into the surface active film during initial bubble expansion occurred at a surface tension of approximately 35 mN/m for the samples with dipalmitoylated SP-C(Leu) compared to approximately 45 mN/m for the non-palmitoylated peptide (Fig. 4). There was an excess of approximately 2.6 monolayers in the surface reservoir for the samples with 2% dipalmitoylated SP-C(Leu), and 2.2 monolayers for the non-palmitoylated peptide, as determined by comparing the surface areas at minimum surface tension at the last film compression with that of the first compression (Fig. 4A,B). For the dipalmitoylated SP-C(Leu), the minimum and very stable surface tension reached upon all of the compressions was equal to or below 1 mN/m, and for the first three compressions, a film area reduction of approximately 20% was required. At the last compression, when the reservoir was nearly exhausted, an area change of approximately 30% was required to reach the minimum surface tension from the equilibrium surface tension of 24 mN/m (Fig. 4B).

In contrast, the minimum surface tension for the successive compressions of the film formed from the non-palmitoylated peptide was approximately 4 mN/m. Lower minimum surface tensions were not possible due to film instability in these experiments, as the bubble suddenly started to assume a more spherical shape with an increasing surface tension to about 25 mN/m and a corresponding decreasing surface area (Fig. 4A). This increase in surface tension during about 0.5–1.0 s occurred at constant bubble volume equal to that reached at minimum surface tension. The changes in surface tension and bubble area were similar to those observed previously during bubble clicks [12], but for the clicks, the changes occurred during a shorter interval in a small fraction

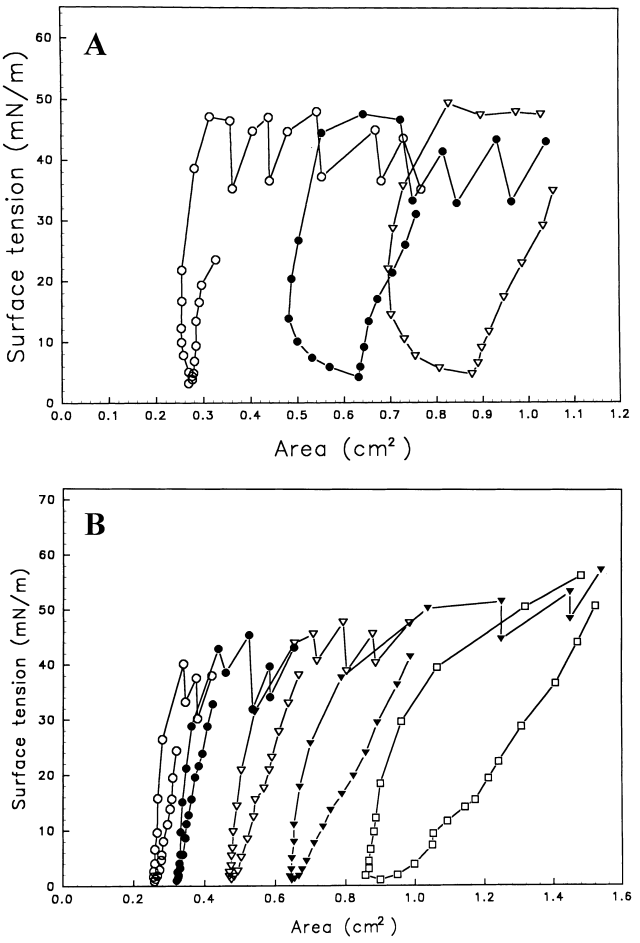

Fig. 4. Series of consecutive quasi-static compression–expansion cycles conducted at increasing bubble areas, after surfactant depletion and replacement of the subphase with buffer (initial bubble formation in 1 mg/ml surfactant). The plots represent consecutive quasi-static cycles, starting with the first cycle at the left. (A) 2% non-palmitoylated SP-C(Leu), (B) 2% dipalmitoylated SP-C(Leu) in DPPC/PG/PA (68:22:9 by mass).

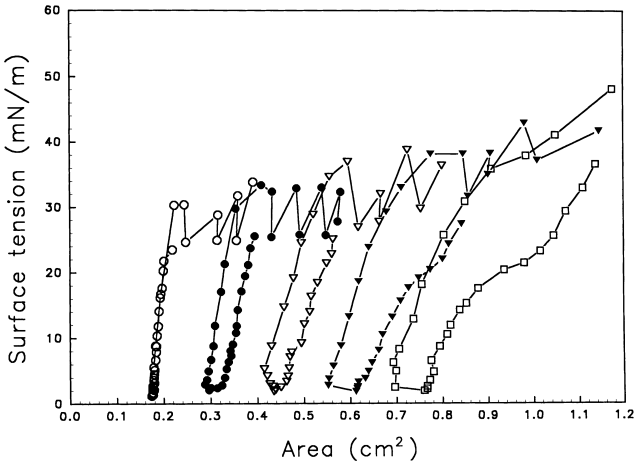

Fig. 5. Series of consecutive quasi-static compression–expansion cycles of BLES, after surfactant depletion and replacement of the subphase with buffer as in Fig. 4.

of a second (~0.1 s). Increasing the concentration of the peptides to 5% neither increased the extent of the reservoir nor facilitated the incorporation of the material from the reservoir into the surface active film (not shown).

The incorporation of surfactant material from the reservoir formed from a suspension of 1 mg/ml bovine lipid extract surfactant (BLES) occurred at surface tension between 25 and 30 mN/m upon stepwise bubble expansion (Fig. 5). There was an excess of 3.2 monolayers in the surface associated reservoir. The film area at minimum surface tension upon the fifth compression was only slightly larger than that on the fourth compression, indicating that nearly all the excess material had moved from the reservoir to the surface active film. The film area compressions required to reach the minimal surface tensions of less than 1 mN/m were approximately 18% for the first three compressions and 23–24% for the fourth and fifth compressions, respectively. This is consistent with the concept that the material incorporated into the surface active film upon bubble expansion remained enriched in DPPC. Although near zero minimal surface tensions were observed for all of the compressions for BLES, the films were slightly less stable than those with dipalmitoylated SP-C(Leu), as indicated by negative slopes upon bubble expansion at low surface tensions (Figs. 4B and 5). However, the incorporation of reserve material occurred at lower surface tensions and there was more excess material in the BLES reservoir.

4. Discussion

In this work, functional effects of palmitoylation of a synthetic analogue of pulmonary surfactant protein C were studied. Surface activity tests, including film formation by adsorption, film compressibility, minimum and maximum surface tensions and stability, were conducted in a captive bubble system on mixtures of DPPC, PG and PA, with dipalmitoylated or non-palmitoylated SP-C(Leu).

The reformation of the surface film during dynamic cycling appears to be influenced by the palmitoyl groups (Fig. 3). With the non-palmitoylated peptide, the dynamic cycles with moderate overcompression at near minimum surface tension depict plateaus of a relatively large compressibility around 20 mN/m (Fig. 3A). In contrast, the compressibility at 20 mN/m of the films containing dipalmitoylated peptide is substantially lower (Fig. 3B, Table 1). In addition, the maximum surface tension, reached upon dynamic cycling, is higher for the films with the non-palmitoylated peptide. These observations suggest that the palmitoyl groups of the peptide affect the organization of the collapse phase generated upon overcompression. The reformation of the film upon area expansion is not as efficient without the palmitoyl groups, and the greater film compressibility as seen by the plateau at ~20 mN/m is consistent with the concept that the films without the palmitoyl groups are less enriched in DPPC. The fact that the film compressibility is affected by the collapse phase generated upon film overcompression has been shown previously [15]. In that work, plateaus at surface tensions below the equilibrium of ~25 mN/m were shown to be generated by film overcompression at minimum surface tension, such that the films appeared to be less enriched in DPPC during cyclic film compression.

Palmitoylation appears to make no discernible difference in the initial lipid film formation, but the amount of the added peptide had a substantial effect. Film formation was better with 2% than with 5% SP-C(Leu), regardless of whether or not the peptide was dipalmitoylated (Fig. 1, Table 1). Interestingly, the concentration effect was more pronounced for the dipalmitoylated than for the non-palmitoylated peptide. The ability of bovine SP-C to accelerate adsorption was compromised by chemical depalmitoylation

[6,7]. This is in contrast to our findings with the synthetic SP-C(Leu) analogue, where palmitoylation of the peptide did not have a discernible influence on de novo film formation. The different activities of chemically depalmitoylated SP-C and non-palmitoylated SP-C(Leu) in de novo film formation might be related to the different secondary structures of the peptides. Depalmitoylated SP-C is less helical than the native peptide [3,6], while non-palmitoylated and dipalmitoylated SP-C(Leu) exhibit similar helical contents [8,10]. There was no discernible difference in the capacity to reach a near zero and stable minimum surface tension under quasi-static conditions, as measured by the film area compression required, between lipid mixtures with dipalmitoylated or non-palmitoylated bovine SP-C [7]. In contrast, in the present study, the lipid mixtures with non-palmitoylated SP-C (Leu) were mechanically less stable at tensions below 5 mN/m as seen by bubble clicks in the surface tension range below 5 mN/m (Fig. 2).

The film area compressions required to reach minimum surface tensions below 5 mN/m are similar whether or not native SP-C [7] or SP-C (Leu) in the dipalmitoylated or non-palmitoylated form is added to the lipid mixtures. Films from pure lipid mixtures of DPPC and egg PG could be compressed to near zero and stable minimum surface tensions, but the film area compressions required were greater than 60% [7]. So, the addition of native SP-C or of SP-C (Leu) in their dipalmitoylated or non-palmitoylated form has a dramatic effect on the capacity to reach near zero tensions, expressed as film area compression required. Thus, films from lipid mixtures in the presence of SP-C appear enriched in DPPC upon film formation, as only films which are enriched in DPPC can achieve near zero and stable minimum surface tensions upon moderate film area compressions [15].

Our experiments clearly demonstrate that the presence of dipalmitoylated SP-C(Leu) mechanically stabilizes the lipid films. Upon quasi-static compression, bubbles stabilized with films containing dipalmitoylated SP-C(Leu) do not click upon compression towards minimum surface tension, such that they can reach lower minimum tensions especially upon the first quasi-static compression (Fig. 2A). These films appear to be enriched in DPPC upon film formation, as seen by the low compressibility at the first quasi-static compression. In contrast, the films with 2% non-palmitoylated SP-C(Leu) show high compressibility during the first cycle, but improve their quality upon repeated quasi-static cycling, as seen by the lower minimum surface tension reached upon the fourth compression and the reduced surface tension–area hysteresis (Fig. 2B). Greater film stability is also seen from the depletion experiments with the dipalmitoylated peptide. Surfactant material incorporated by adsorption steps into the surface active film from the surface reservoir appears to remain enriched in DPPC when palmitoyl groups are present, as bubble clicking is absent and near zero surface tensions can be achieved upon repeated cycling (Fig. 4B). In contrast, the films with the peptide lacking the palmitoyl groups become unstable as seen by bubble clicking at low surface tension and by the inability to reach surface tensions of $\sim$1 mN/m. (Fig. 4A). Furthermore, the depletion experiments also show that the incorporation of reservoir material occurs at lower surface tensions upon the initial expansion steps for the films with the dipalmitoylated peptide. However, the lipid incorporation reached by BLES cannot be matched by the lipid mixtures with the SP-C analogue (Fig. 5). BLES contains both SP-B and SP-C, as well as a different lipid mixture than the one now used in combination with SP-C(Leu). It appears that in addition to SP-C or analogues thereof, addition of SP-B, or analogues thereof, and/or modification of the lipid mixture is needed for optimal surface activity. In particular, further work is required to reveal the influence of the lipid mixture per se on the surface properties, and the activity of dipalmitoylated SP-C(Leu) versus non-palmitoylated SP-C(Leu) in different lipid mixtures.

The molecular basis for the apparent effects of the palmitoyl groups is not known. The palmitoyl chains may have the ability to link the air–water monolayer to the adjacent bilayer and to link two bilayers together. This would promote the formation of the surface associated surfactant reservoir and the mechanical stability of the complex consisting of the monolayer and stacks of bilayers associated with the monolayer [17,18]. These bilayer stacks are likely linked together by the combined action of the palmitoylated SP-C [19] and the fusion promoting SP-B [20]. The palmitoyl groups may also affect the activity of SP-C(Leu) by modulation of peptide–peptide

interactions. Non-palmitoylated SP-C(Leu) readily forms dimers and higher order oligomers [8], but the dipalmitoylated peptide mainly forms monomers and dimers (M. Gustafsson and J. Johansson, unpublished). Further work is necessary in order to fully understand the basis for the palmitoyl-mediated effects, and the respective function of SP-B and SP-C.

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